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Endogenous calmodulin interacts with the epidermal growth factor receptor in living cells

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Abstract **We have previously shown that exogenous calmodulin (CaM) binds to the epidermal growth factor receptor (EGFR) at its cytosolic juxtamembrane region inhibiting its tyrosine kinase activity. We demonstrate in this report that endogenous CaM binds to EGFR in intact cells as CaM co-immunoprecipitates with EGF-activated and non-activated receptors. We also show in living cells that cell-permeable CaM inhibitors prevent the full transphosphorylation of wild type EGFR but not the transphosphorylation of an insertional EGFR mutant in which the CaM-binding domain was divided into two parts. Overall these results suggest that CaM interacts with EGFR in vivo.**

Key words: Calmodulin; Epidermal growth factor receptor; Living cell

1. Introduction

An early signal generated by the activation of epidermal growth factor receptor (EGFR) upon ligand binding is a transient increase in the cytosolic concentration of free calcium ion ($[Ca^{2+}]_{cyt}$) (see for review [1]). Entry of extracellular Ca^{2+}, and Ca^{2+} release from intracellular stores, both appear to contribute to the generation of the EGF-mediated $[Ca^{2+}]_{cyt}$ spike [2–4]. Despite being a short-lived event, this process has great importance for the functioning of the receptor, as it is relevant for the inhibition of its tyrosine kinase activity, abating in this manner subsequent downstream signaling. In addition to promoting the activation of protein kinase C, which phosphorylates Thr654 at the EGFR inducing its downregulation [5–8], this Ca^{2+} surge facilitates the formation of the Ca^{2+}/calmodulin (CaM) complex [9]. This complex in turn activates calmodulin-dependent protein kinase II (CaMK-II) further phosphorylating Ser1046/Ser1047 at the EGFR, contributing in this manner to the inhibition of the receptor tyrosine kinase activity [10,11].

In addition to the indirect regulation of EGFR by the Ca^{2+}/CaM complex because of the activation of CaMK-II, our group has shown that CaM directly binds to this receptor at its cytosolic juxtamembrane region (Arg645–Gln660) in a Ca^{2+}-dependent manner, inducing the inhibition of its tyrosine kinase activity [12–16]. The interaction of the Ca^{2+}/CaM complex at the cytosolic juxtamembrane region of the receptor has been confirmed by others [17]. Likewise, we have demonstrated that the Ca^{2+}-dependent binding of CaM to this EGFR segment prevents the phosphorylation of Thr654 by protein kinase C, and conversely the phosphorylation of this residue prevents CaM binding [14].

Despite these accumulated observations, no evidence was available until now on the direct interaction of endogenous CaM with EGFR, although CaM has being implicated in the regulation of the intracellular sorting and trafficking of the receptor in intact cells [18]. In this report we fill this gap by showing that endogenous CaM can be co-immunoprecipitated with EGFR from two different cell lines overexpressing this receptor, and that cell-permeable CaM antagonists partially inhibit the EGF-dependent transphosphorylation of wild type EGFR but not the transphosphorylation of an EGFR mutant in which its CaM-binding domain was split into two parts by the insertion of a non-relevant sequence. This suggests that a CaM/EGFR complex is present in living cells, and that the described CaM-binding domain [12,14,15,17] has indeed a functional role in vivo.

2. Materials and methods

2.1. Reagents

Monoclonal anti-CaM antibody developed in mouse (recognizing the carboxy-terminal amino acids 128–148 of human CaM) was obtained from Upstate Biotechnology. Monoclonal anti-EGFR antibodies (clone 13 recognizing the intracellular segment 996–1022, and clone 528 recognizing the extracellular domain of the human receptor) developed in mouse, and mouse monoclonal anti-phosphotyrosine RC20 antibody conjugated to horseradish peroxidase were obtained from Transduction Laboratories. Anti-mouse immunoglobulin G (IgG) (Fc-specific) developed in goat and conjugated to horseradish peroxidase, deoxycholic acid (sodium salt), Triton X-100, Tween 20, Fast green FCF, bicinchoninic acid, copper sulfate, sodium orthovanadate, leupeptin, pepstatin A, aprotinin, and phenylmethylsulfonyl fluoride

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Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic concentration of free calcium ion; CaM, calmodulin; CaMK-II, calmodulin-dependent protein kinase II; EGF, epidermal growth factor; EGFR, EGF receptor; EGTA, [ethylene bis(oxyethylenenitrilo)]-tetraacetic acid; FBS, fetal bovine serum; IgG, immunoglobulin G; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W12, *N*-(4-aminobutyl)-1-naphthalenesulfonamide; W13, *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide

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(PMSF) were purchased from Sigma. Human recombinant EGF was obtained from PeproTech EC, and the prestained molecular mass standards for electrophoresis were from Bio-Rad. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), *N*-(4-aminobutyl)-1-naphthalenesulfonamide (W12), and *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide (W13) were from Calbiochem, and BioTrace™ polyvinylidene difluoride (PVDF) membranes were purchased from Pall Gelman Laboratory. The enhanced chemiluminescence Luminol (ECL™) reagents were from Amersham Pharmacia Biotech. Other chemicals used in this work were of analytical grade.

2.2. Cell cultures

Murine EGFR-T17 fibroblasts, a stably transfected cell line overexpressing the human EGFR [19] (donated by Dr. J.M. Mato, Instituto de Investigaciones Biomédicas, Madrid, Spain), human epidermoid carcinoma A431 cells overexpressing EGFR [20] (donated by Dr. R. Perona, Instituto de Investigaciones Biomédicas, Madrid, Spain), RI1 fibroblasts, a stably transfected cell line expressing a human EGFR mutant with the insertion of a non-relevant highly acidic 23 amino acid sequence between Arg647 and His648 dividing into two segments the CaM-binding domain [21] (donated by Dr. A. Sorokin, Medical College of Wisconsin, Milwaukee, WI, USA), and N7xHERc fibroblasts, a stably transfected cell line expressing the wild type human EGFR (donated by Dr. A. Ullrich, Max-Planck-Institut für Biochemie, Martinsried, Germany), were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and 40 μg/ml gentamicin in a humidified atmosphere of 5% (v/v) CO_2 in air at 37°C. The cells were maintained overnight in an FBS-free medium before performing the experiments.

2.3. Co-immunoprecipitation of CaM with EGFR

Serum-starved confluent A431 tumor cells and EGFR-T17 fibroblasts grown in 10 dishes of 15 cm diameter containing 10 ml of culture medium were incubated in duplicate for 1 min at room temperature in the absence and presence of 10 nM EGF or at the concentrations indicated in the legends of the figures. Thereafter, the medium was removed and 1 ml of a lysis buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM [ethylene bis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), 0.3% (w/v) Triton X-100, 0.16% deoxycholic acid, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin was added and incubated for 30 min on ice. The cell extract was collected by centrifugation on a bench-top centrifuge at $15\,600 \times g_{max}$ for 35 min, and 1.5 mM $CaCl_2$ was added to the supernatant. Solubilized proteins (2–5.2 mg) were incubated for 3–12 h in 200 μl of a medium containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM EGTA, 1.5 mM $CaCl_2$ (0.5 mM free Ca^{2+}), 1 mM sodium orthovanadate, 1 mM PMSF, and 1.5 μg anti-EGFR antibody (recognizing the extracellular domain) precoupled to 30 μl slurry of protein A-agarose. The beads were collected by low-speed centrifugation and washed six times in a buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM sodium orthovanadate, and 1 mM PMSF. Mock immunoprecipitation experiments were performed using 1.5 μg of a non-relevant mouse IgG fraction. The samples were boiled for 5 min in Laemmli sample buffer, the beads were removed by low-speed centrifugation, and the supernatant processed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) and Western blot analysis as described below.

2.4. CaM antagonist treatment and activation of EGFR in living cells

Cells grown to confluence in 6 well culture dishes containing 2 ml of culture medium, and deprived of FBS overnight, were incubated for 15 min at room temperature with the CaM antagonists W7, W12 or W13 at the concentrations indicated in the figures. Thereafter, 10 nM EGF was added and the incubation was continued for different periods of time (30 s to 30 min) as indicated in the figures, and the reaction was arrested upon addition of 10% (w/v) trichloroacetic acid. The precipitated proteins were boiled for 5 min in Laemmli sample buffer. The tyrosine phosphorylation level of EGFR was determined after SDS–PAGE and Western blot analysis using an anti-phosphotyrosine antibody as described below.

2.5. Electrophoresis and immunoblot analysis

Slab gel electrophoresis was performed according to Laemmli [22] at 12 mA overnight in a linear gradient 5–20% (w/v) polyacrylamide gel in the presence of 0.1% (w/v) SDS at pH 8.3. Proteins in the gel were electrotransferred to a PVDF membrane in a medium containing 48 mM Tris base, 36.6 mM glycine, 0.04% (w/v) SDS, and 20% (v/v) methanol, and the proteins were fixed with 0.2% (v/v) glutaraldehyde for 45 min in a medium containing 0.1% (w/v) Tween 20, 100 mM Tris–HCl (pH 8.8), 0.5 M NaCl, and 0.25 mM KCl (T-TBS medium). The PVDF membrane was blocked with 5% (w/v) bovine serum albumin in T-TBS medium and probed with the anti-phosphotyrosine antibody conjugated to horseradish peroxidase (1/2000 dilution). The upper part of the PVDF membrane was stripped in a medium containing 100 mM β-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris–HCl (pH 6.7) at 50°C for 30 min, or after overnight incubation at room temperature, and reprobed with the anti-EGFR antibody recognizing the intracellular domain (1/2500 dilution). The lower part of the PVDF membrane was probed with the anti-CaM antibody (1/1000 dilution). Anti-mouse IgG coupled to horseradish peroxidase (1/3000 dilution) was used as secondary antibody in both instances. The positive bands were developed, after appropriate time exposure, using the ECL™ method following instructions from the manufacturer. When required, the intensity of the phosphotyrosine-containing EGFR band was quantified with a computer-assisted scanning densitometer using the NIH Image 1.59 program. Corrections were made for the amount of protein present in the electrophoretic tracks as detected by Fast green FCF staining after densitometric reading.

2.6. Protein determination

The protein concentration was determined using bicinchoninic acid and copper sulfate (BCA method) using standards of bovine serum albumin as described [23].

3. Results

3.1. CaM co-immunoprecipitates with EGFR

To test whether endogenous CaM directly interacts with EGFR, we prepared solubilized whole cell extracts from non-stimulated EGFR-T17 fibroblasts and A431 tumor cells, and performed immunoprecipitation experiments in the pres-

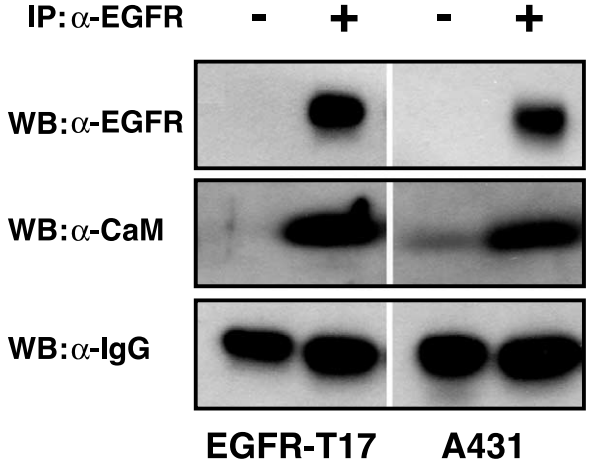

Fig. 1. Endogenous CaM co-immunoprecipitates with EGFR. Solubilized whole cell extracts from EGFR-T17 fibroblasts (3 mg protein) and A431 tumor cells (5.2 mg protein) were subjected to immunoprecipitation (IP) using an anti-EGFR antibody (α-EGFR). Mock immunoprecipitation experiments using a non-relevant mouse IgG fraction instead of the anti-EGFR are also presented. The samples were separated by SDS–PAGE, electrotransferred to a PVDF membrane, and the upper and lower parts of this membrane were probed, respectively, with anti-EGFR (α-EGFR), and anti-CaM (α-CaM) antibodies by Western blot analysis (WB). Loading controls showing the amount of IgG present in the samples as detected using the secondary anti-mouse IgG antibody (α-IgG) coupled to peroxidase are also presented. The apparent molecular masses of the different bands were as follows: EGFR 170 kDa, CaM 21 kDa (because EGTA present), and IgG 55 kDa. Representative experiments of seven performed in EGFR-T17 fibroblasts and six performed in A431 tumor cells are presented.

ence of Ca^{2+} using an anti-EGFR antibody (α-EGFR). Fig. 1 shows that the immunoprecipitated material from both cell lines gave a positive signal when probed with an anti-CaM antibody (α-CaM) using Western blot analysis. A faint CaM signal was detected in the material derived from mock immunoprecipitations using a non-relevant mouse IgG fraction instead of the anti-EGFR antibody. This latter spurious signal, which does not interfere with our results, could be the consequence of non-specific binding of trace amounts of CaM to the protein A-agarose beads used to pull down the immune complex, and it was more noticeable in A431 tumor cells than in EGFR-T17 fibroblasts. As expected, controls reveal that EGFR was present in the immunoprecipitated material but not in mock samples when probed using an anti-EGFR antibody (α-EGFR). To ascertain that comparable amounts of sample were analyzed in each lane, we show the IgG signal detected in the immune complexes using the secondary anti-IgG antibody (α-IgG) coupled to peroxidase.

Our previous results demonstrated that the interaction of exogenous CaM with EGFR was not significantly affected by the degree of tyrosine phosphorylation of the receptor [16]. To determine if this is also the case for the interaction of endogenous CaM with EGFR, we performed co-immunoprecipitation experiments using EGFR-T17 and A431 cells that were previously exposed to increasing concentrations of EGF for 1 min in order to transphosphorylate the receptor at its maximum level.

The results in Fig. 2 clearly show that increasing concentrations of EGF up to 50 nM progressively increase the tyrosine phosphorylation of the immunoprecipitated EGFR from EGFR-T17 fibroblasts as determined by Western blot using an anti-phosphotyrosine antibody (α-P-Tyr). In contrast, the phosphorylation level of the immunoprecipitated receptor from A431 tumor cells was already high in the absence of EGF, and little increase was observed when cells were stimulated with 50 nM EGF. Reprobing the stripped PVDF membrane with an anti-EGFR antibody (α-EGFR) shows that comparable amounts of receptor were present in the different samples, except in mock immunoprecipitation experiments using a non-relevant mouse IgG fraction instead of the anti-EGFR antibody. In all cases, however, we show that the signal corresponding to CaM as probed with an anti-CaM antibody (α-CaM) was invariant. This demonstrates that the degree of activation of the EGFR, as inferred from its tyrosine phosphorylation status, does not significantly modify the extent of binding of endogenous CaM to the receptor, in agreement with our previous results using exogenous CaM [16]. We again confirm in Fig. 2 that little, if any, CaM signal was detected in mock immunoprecipitation experiments. As previously indicated, the IgG signal detected in the immune complexes using the secondary anti-IgG antibody (α-IgG) coupled to peroxidase is also shown to ascertain that a similar amount of samples were present in each lane.

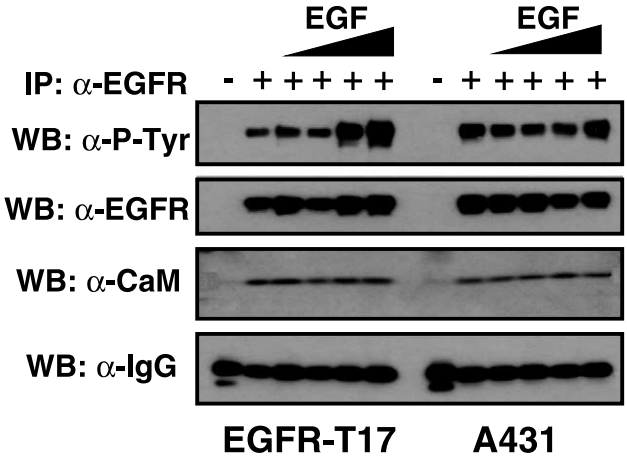

Fig. 2. Activation of EGFR does not modify the binding of endogenous CaM to the receptor. Solubilized whole cell extracts from EGFR-T17 fibroblasts (2 mg protein) and A431 tumor cells (3.1 mg protein) from non-stimulated cultures (lanes 1 and 2) and cultures stimulated with increasing concentrations (0.1, 1, 10, and 50 nM) of EGF for 1 min (lanes 3–6) were subjected to immunoprecipitation (IP) using an anti-EGFR antibody (α-EGFR). Mock immunoprecipitation experiments using a non-relevant mouse IgG fraction instead of the anti-EGFR are also presented. The samples were processed as in Fig. 1 and probed, respectively, with anti-phosphotyrosine (α-P-Tyr), and anti-CaM (α-CaM) antibodies. The upper part of the PVDF membrane was also stripped and reprobed with the anti-EGFR (α-EGFR) antibody. Loading controls showing the amount of IgG present in the samples as detected using the secondary anti-mouse IgG antibody (α-IgG) coupled to peroxidase are also presented. The apparent molecular masses of the different bands were as follows: phosphorylated and total EGFR 170 kDa, CaM 21 kDa (because EGTA present), and IgG 55 kDa. Representative experiments of two performed in each cell line are presented.

3.2. *Cell-permeable CaM antagonists prevent full EGFR activation in intact cells*

To obtain information about the possible functional role of endogenous CaM on EGFR, we incubated intact cells with a set of structurally related cell-permeable CaM antagonists but with increasing affinities for this Ca^{2+} regulator ($W12 \ll W13 < W7$), and determined the degree of EGF-induced transphosphorylation of the receptor after treatment with these compounds. We performed these experiments in N7xHERc fibroblasts, a transfected cell line expressing the human EGFR in lesser extent than EGFR-T17 fibroblasts. Fig. 3A shows that increasing concentrations of W7 and W13 significantly prevented the full transphosphorylation of EGFR, reaching a CaM antagonist-resistant plateau (approximately 50–60% inhibition) at approximately 40 μM, while W12 had a far lesser inhibitory effect. Moreover, the degree of inhibition of EGFR transphosphorylation inversely correlates with the average inhibition constant (IC_{50}) of these compounds for CaM expressed in a logarithmic scale (see Fig. 3B), suggesting that this effect was indeed due to the inhibition of endogenous CaM. Moreover, we have calculated that the average IC_{50}s for EGFR transphosphorylation were 12 μM for W7, 33 μM for W13, and >100 μM for W12, well within the range described for other systems. We have previously determined that W7, W13, and W12 do not have any inhibitory action on the tyrosine kinase activity of a purified EGFR preparation (Ruano, Salas and Villalobo, unpublished results), further suggesting that the observed effect in intact cells was due to endogenous CaM inactivation.

3.3. *An insertional EGFR mutant at its CaM-binding domain is resistant to CaM antagonist action*

To determine whether the described CaM-binding domain of EGFR, comprising Arg645–Gln660 [12,14,15,17], participates in the regulation of EGFR transphosphorylation in living cells, we performed the following experiments. We studied the effect of W13 on the activation of both wild type EGFR

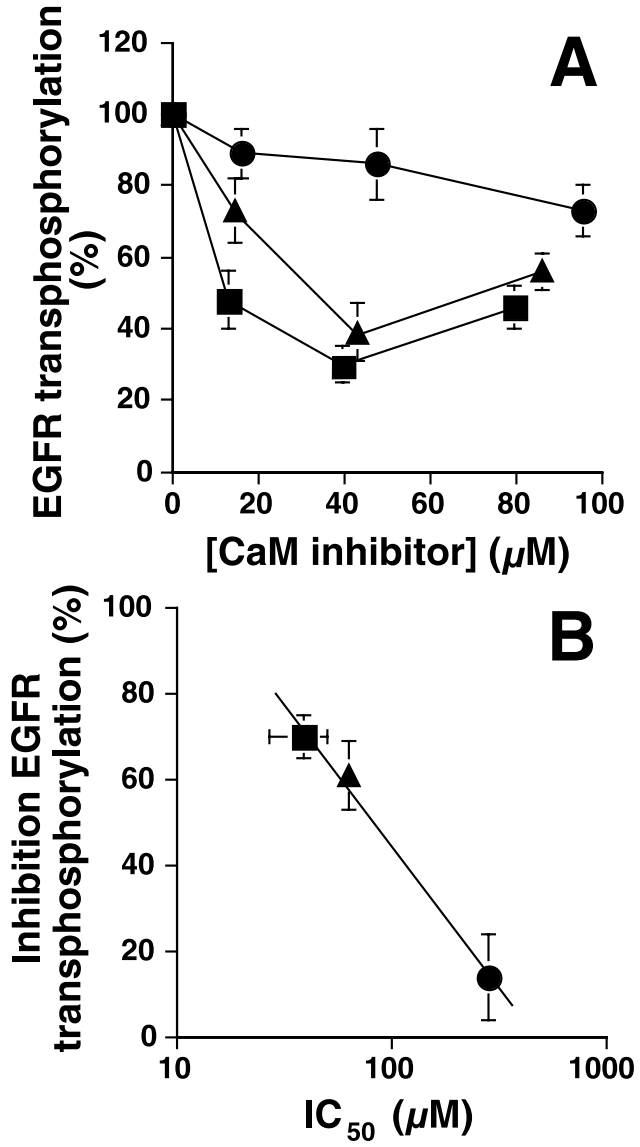

Fig. 3. Cell-permeable CaM antagonists prevent full EGFR transphosphorylation. A: N7xHERc fibroblasts were treated with the indicated concentrations of W12 (circles), W13 (triangles) or W7 (squares) for 15 min, and thereafter 10 nM EGF was added for 1 min. Transphosphorylation of EGFR was determined by Western blot using an anti-phosphotyrosine antibody and quantified as described in Section 2. The plot presents the average ± S.D. percent phosphorylation level of EGFR from three independent experiments. B: The plot presents the average ± S.D. percent inhibition of EGFR transphosphorylation ($n = 3$, vertical error bars) by 47 μM W12 (circles), 43 μM W13 (triangles) or 40 μM W7 (squares) for 15 min, against the average ± S.D. inhibition constant (IC_{50}) of these compounds for CaM ($n = 2$, horizontal error bars), as determined by the manufacturer, inhibiting the activity of two distinct Ca^{2+}/CaM-dependent enzymes, cyclic nucleotide phosphodiesterase and myosin light chain kinase. The smallest horizontal error bars are hidden within the symbols.

and an EGFR mutant in which the CaM-binding domain was divided into two segments by the insertion of a non-relevant highly acidic sequence of 23 amino acids between Arg647 and His648 [21]. To perform these experiments, we used N7xHERc and RI1 fibroblasts expressing, respectively, the wild type and the mutant EGFR. These cell lines express comparable numbers of receptors per cell as suggested by their similar phosphorylation levels.

Fig. 4A shows a time course of EGF-dependent tyrosine phosphorylation of wild type EGFR from N7xHERc fibroblasts reaching a maximum at 1–2 min, followed by a rapid partial dephosphorylation ($t_{1/2} \approx 4$ min), that reaches an apparent steady state at 50% phosphorylation after 15 min, remaining roughly constant for up to 1 h (not shown). The presence of W13 does not prevent the initial rapid EGFR phosphorylation burst, but reaches a maximum only at half the value of its control in the absence of CaM antagonist. As

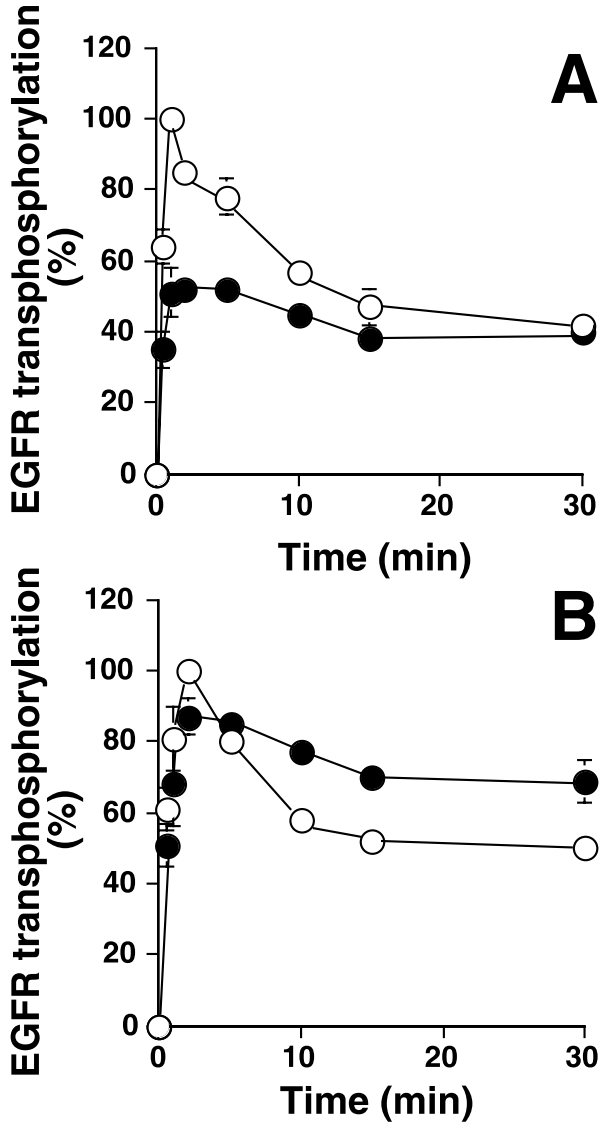

Fig. 4. Disruption of the CaM-binding domain in an EGFR mutant results in resistance to CaM antagonist treatment. N7xHERc fibroblasts (A) and RI1 fibroblasts (B), expressing respectively the wild type and mutant EGFR, were incubated in the absence (open symbols) and presence (filled symbols) of 43 μM W13 for 15 min, and thereafter 10 nM EGF was added for the indicated period of time. Transphosphorylation of EGFR was determined by Western blot using an anti-phosphotyrosine antibody and quantified as described in Section 2. The plot presents the average ± S.D. percent phosphorylation level of wild type (A) and mutant (B) EGFR from two (A) or four (B) independent experiments. The smallest error bars are hidden within the symbols.

can be observed, the dephosphorylation of EGFR was greatly reduced in the presence of W13.

When similar experiments were performed using RI1 fibroblasts, expressing the mutant EGFR with a disrupted CaM-binding domain (see Fig. 4B), the phosphorylation/dephosphorylation kinetics of the receptor in the absence of W13 was almost identical to that of wild type EGFR (compare open symbols in Fig. 4A,B). In contrast, the maximum level of tyrosine phosphorylation of the mutant EGFR in W13-treated cells reached nearly the same value as in the absence of W13. We also noticed that the subsequent dephosphorylation of the mutant EGFR was greatly reduced in the presence of W13 as compared with that in the absence of this drug, similar to what was observed in wild type EGFR (compare filled symbols in Fig. 4A,B).

4. Discussion

We have shown in this report that endogenous CaM binds to EGFR as demonstrated by co-immunoprecipitation experiments, suggesting that a CaM/EGFR complex is present in living cells. However, the immune detection of different proteins probed with diverse antibodies against distinct antigenic determinants hardly can be compared to adequately quantify the amount of two unrelated proteins present in a sample, as this reaction greatly depends on non-comparable affinities of primary plus secondary antibodies for their respective targets. Therefore, we could not determine in our experiments the actual CaM/EGFR stoichiometry in the co-immunoprecipitated complex. In view, however, of the large amount of protein needed for immunoprecipitation in order to detect a readable CaM signal, and because both EGFR-T17 and A431 cell lines used in our experiments overexpress EGFR [19,20], we suggest that this stoichiometry is probably low. If this were the case, only a fraction of EGFR would be coupled to CaM in living cells at a given time.

In general, comparing the immunoprecipitated EGFR from non-treated and EGF-treated cells, we observed better EGF-dependent stimulated phosphorylation of the receptor from EGFR-T17 fibroblasts than from A431 tumor cells. This could be due to the fact that the latter cell line shows higher levels of basal phosphorylation of EGFR than the former, perhaps because of autocrine/paracrine activation of the receptor by endogenous EGFR ligand(s). The EGF-dependent phosphorylation of EGFR as measured in whole cell extracts from A431 tumor cells was, however, very high (not shown). This suggests, therefore, that the antibody used to immunoprecipitate EGFR could have relatively higher affinity for the phosphorylated receptor than for its non-phosphorylated form.

Although EGFR activation generates a transient increase in the $[Ca^{2+}]_{cyt}$ [1], our results show that the extent of binding of endogenous CaM to EGFR is independent of its tyrosine phosphorylation status. It is important to have in mind that CaM could interact with a distinct yet unknown pool of EGFR molecules, as low-affinity and high-affinity EGFR species are known to be present in the cell. The location of functional EGFR molecules in several subcellular compartments including the plasma membrane [24], endosomes [25], and even the nucleus [26,27], and nucleoli [27] has been reported. This offers the possibility that CaM could interact with the receptor in specialized subcellular regions performing specific tasks.

The precise role of CaM on the functional cycle of EGFR in living cells is yet unknown. However, the implication of this Ca^{2+} sensor in the modulation of the receptor tyrosine kinase activity at the plasma membrane [12,13], during its internalization [14], intracellular sorting and trafficking [18], and even during its nuclear translocation [15,17,27] has been demonstrated and/or suggested. In the latter case, this is supported by the fact that the reported CaM-binding domain and the nuclear localization sequence of EGFR have overlapping sequences [14,17,26,27]. Our results demonstrate that CaM indeed has a regulatory role on EGFR in living cells, as using cell-permeable CaM antagonists we observed a depletion of EGF-activatable receptors in the cells, and W7 has been shown to inhibit EGF-induced, but not EGF-independent, proliferation of a human hepatoma cell line [28]. Because W13 promotes the sequestration of EGFR at endosomes [18], the amount of EGF-responsive receptors at the cell surface would be expected to decrease upon treatment with this CaM antagonist, perhaps explaining in this manner the decreased tyrosine phosphorylation of EGFR observed in our experiments.

Although calcineurin has been shown to dephosphorylate phosphotyrosine residues of EGFR in vitro [29,30], little is known about the implication of this CaM-dependent phosphoserine/threonine phosphatase on the dephosphorylation of EGFR in intact cells. Our results show, however, that inhibition of endogenous CaM with W13 relents EGFR dephosphorylation, suggesting that calcineurin could directly or indirectly be involved in the process.

The cytosolic juxtamembrane region of EGFR has been implicated in the control of multiple receptor functions [15], including those mediated by the binding of CaM to this region, more precisely between residues Arg645 and Gln660 [14,15,17]. Although the identification of this CaM-binding domain in EGFR represented a significant advance in our understanding of the functionality of this receptor, the involvement of this site in the binding of CaM in living cells has not been previously determined. Our results now show for the first time that disruption of the positively charged amphiphilic helical structure of the CaM-binding domain of EGFR in the insertional mutant used renders the receptor mostly insensitive to W13 action. Hence, the receptor remains nearly fully activatable by EGF in the presence of this CaM antagonist, although its subsequent dephosphorylation is significantly blocked. The reason for this manifold behavior is not yet fully understood, as several CaM-dependent systems with distinct affinities for CaM are notoriously operative on EGFR. Thus, phosphorylation of the receptor by CaMK-II, which is a high-affinity system [10,11], and direct binding of CaM to EGFR, which is a low-affinity system [12,14,16], are at work. However, one possibility that could explain these results is that a significant, but yet partial, decrease of active cellular CaM subjects the wild type EGFR prone to endosome sequestration as proposed [18]. Therefore, the high-affinity CaMK-II systems could still be in operation, while the absence of a functional CaM-binding domain in the mutant EGFR retains this receptor at the cell surface, and therefore remains able to undergo EGF-dependent activation. This is in agreement with our suggestion that binding of CaM to EGFR may be required for its internalization [14]. We have also observed in cells overexpressing EGFR, such as EGFR-T17 and A431, that the inhibitory action of W13 on EGF-depen-

dent receptor phosphorylation was only observed in the presence of the Ca^{2+} ionophore A23187 (Ruano and Villalobo, unpublished results). This suggests that the increase in $[Ca^{2+}]_{cyt}$ generated upon EGF stimulation in the absence of the ionophore was low enough in these cell lines.

Because disparate signals converge on EGFR to dictate their function and fate, and the CaM-dependent mechanisms directly or indirectly acting on the receptor appear to be multiple, the study of CaM-regulated EGFR functions in living cells is highly complex and laborious to analyze. Therefore, additional work should be performed to determine as a leading priority the subcellular location of EGFR species able to interact with CaM. The use of various technologies to monitor real-time protein–protein interactions in living cells could help in this task.

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